

INTERANNUAL DIFFERENCES IN THE ESTUARINE GHOST SHRIMP,
NEOTRYPAEA CALIFORNIENSIS

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by

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INTERANNUAL DIFFERENCES IN THE ESTUARINE GHOST SHRIMP,
NEOTRYPAEA CALIFORNIENSIS

by

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ABSTRACT

INTERANNUAL DIFFERENCES IN THE ESTUARINE GHOST SHRIMP, *NEOTRYPAEA CALIFORNIENSIS*

By

Michael Buncic

The dispersal of invertebrate marine larvae can be expected to be wide ranging and show little population structure. *Neotrypaea californiensis*, the burrowing ghost shrimp, is found throughout the waters and coastal estuaries of the northwestern United States. Three hundred and four larval samples were used to study population diversity and structural difference occurring over the course of spawning periods from June to September in successive years (2005 and 2006). Data and genetic analysis from nucleotide sequencing of a section of the mitochondrial Cytochrome C oxidase subunit I (COI) gene suggest that ocean-borne larvae off the coast of Oregon and Washington show little barrier to dispersal or gene flow in the open ocean. There was evidence of significant temporal differences in the genetic composition in the oceanic larval populations. Larvae from 2005 and 2006 formed samples that were genetically distinguishable from one another. Larvae collected in 2006 inside the Yaquina Bay estuary showed significant genetic distance from larvae in the offshore pool.

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INTRODUCTION

The mud shrimp *Neotrypaea californiensis* (Family thalassinidae) is a burrowing shrimp found along the Pacific Coast within estuary mudflats from Alaska to Baja California. The burrows often reach a depth of 50 cm and have multiple openings to the surface. Mating and reproduction are not well understood. From April to August larvae are released into tidal flow and leave the estuary to enter the offshore ocean currents. Five larval stages develop over a period of six to eight weeks. Larvae then return to estuaries during flood tides ranging from August to October (Dumbauld et al. 1996). Larvae that eventually develop into adult shrimp have a lifespan that may range from four or five years to possibly longer.

The ghost shrimp is capable of populating estuary mud flats at high density. This has significant effect on the ecosystem within the area which they burrow (Dumbauld et al. 1996; Feldman et al. 1997). The community is influenced by the high amount of sediment which the burrowing produces. Species which are intolerant of such conditions will suffer. Dungeness crabs, *Cancer magister*, may be threatened in part by *Neotrypaea californiensis* and another thalassinid shrimp, *Upogebia pugettensis* (Feldman et al. 1997). Shellfish aquaculture within the coastal areas of Oregon undergoes pressure due to the burrowing and sediment effect on oyster population. Survival of oysters is diminished by sinking of larvae within burrows and sediment of the ghost shrimp. An active mitigation effort has been employed, both by using pesticide and by placing oyster shell over the mud flat regions to alter substrate selection (Feldman et al. 1997).

The purpose of this study is to examine temporal variation in the genetic make-up of *Neotrypaea californiensis* larvae dispersed off the Oregon and Washington coastlines. Additionally, I examine the influence of oceanic barriers on gene flow and genetic distance in order to test my hypothesis that variance in ocean currents results in year-to-year variation in the genetic make-up of the larvae. The inclusion of a sample set of estuary larvae of the same season is intended to compare differences with the ocean-bound larvae and possibly infer a relationship as to any resulting recruitment.

The term “phylogeography” was first used as a description of geographically structured intraspecific genealogies (Avice et al. 1987; Dawson 2001). A previous associated study, conducted as a master’s thesis at San Jose State University (Kozuka 2008), has examined the phylogeography of *N. californiensis* larvae within the open ocean off the Oregon coast. Little evidence was found to support any reduction in gene flow or significant genetic distance between offshore sampling sites ranging up and down the coast.

The planktonic larvae of many invertebrate species undergo wide-ranging dispersal, even across transoceanic distances. This observation was the subject of several foundational papers in the field (Scheltema 1971, 1988). Molecular investigation of these dispersal patterns led to an understanding that limited genetic distance can exist even across great geographical distance, including along coastlines (Diaz-Ferguson et al. 2009; Palumbi 1994). Larvae released from estuaries can undergo ocean travel durations which vary from weeks to months. Various factors may limit this dispersal and produce genetic

breaks not foreseen (Barber et al. 2002; Dawson 2001; Pernet et al. 2008; Sotka et al. 2004).

Increasing the duration of time that larvae spend in ocean travel has been correlated to increased dispersal distance (Shanks et al. 2003) and gene flow (Dawson 2001; Hedgecock 1986). Dawson (2001) described a relationship in which higher planktonic duration can limit phylogeographic structure. Dawson (2001) also highlights the fact that fecundity and habitat isolation have a direct effect on the structure of the populations. High fecundity tends to reduce the phylogeographic structure. Populations isolated by oceanographic barriers tend to have high structure, while ocean-traveling populations tend to have low structure. Retention of larvae near an estuary can be beneficial, as this insures that some portion of the population returns to the region of origin. This may lead to a reduction in gene flow with nearby populations and increased genetic distance (Bilton et al. 2002).

Accordingly, it is expected that long-range dispersal of larvae results in strong gene flow and limited differentiation in the genetic composition of populations occurring along a coastline. The occurrence of genetic breaks within a taxonomic species range is in fact used as evidence of dispersal barriers, which are very often dictated by current patterns (Dawson 2001; Hedgecock 1986; Palumbi 1994). Strong genetic breaks in population structure may occur over even small geographic distances (Barber et al. 2002; Marino et al. 2010).

Coastal oceanography and larval behavior have been shown to alter the dispersal and retention of planktonic larvae (Dawson 2001; Hedgecock 1986; Palumbi 1994; Pernet

et al. 2008). During flood and ebb tidal cycles, larvae may alter their positions within the water column based on the direction of the current (Cronin & Forward 1986; Marta-Almeida *et al.* 2006; Olmi 1994; Yannicelli *et al.* 2006). During flood tides larvae may swim to the upper portion of the column to maintain distances near the continental shelf. During seaward flow, larvae can descend the water column to avoid moving far offshore. Predators also affect water column placement and thus retention, as some larvae may rise within the column during periods when predators are not present (Bollens & Frost 1989).

The topography off the Pacific coast of the western United States has been shown to have a specific effect on both the dispersal of larvae and eventual population structure (Dawson 2001; Pernet et al. 2008; Sotka et al. 2004). The California Current is expected to be the primary means of transport of larvae along the coast of the western United States. During the summer the general direction of flow within the current is southward along the coast, averaging 10 cm/s. The width of the current may reach 1000 km with depths as great as 500 m. During the winter and localized events, flow reverses to a northerly direction. A significant nutrient-rich upwelling current is present near the eastern edge of the flow in the summer months (Gan & Allen 2005; Hickey 1979; Sotka *et al.* 2004). Local upwelling events and costal topology can vary the current flow on local scales (Botsford 2001) which results in local variation in larval recruitment and retention (Yannicelli et al. 2006).

Sotka et al. (2004) used surface drifters to demonstrate that the movement of waters off the Oregon coast may lead to dispersal patterns both northward and southward from their original release point. However it was shown that there is little resulting

exchange of Oregon waters with the waters beyond the northern reaches of California. A number of studies have also demonstrated a reduction in gene flow, resulting in a genetic break between the regions lying to the north and south of Point Conception CA (Dawson 2001; Pernet et al. 2008).

A variety of molecular markers may be employed to examine phylogeographic structure. The mitochondrial gene, Cytochrome C oxidase subunit I (COI), has been previously utilized as a marker within a wide range of studies examining population structure (Barber et al. 2002; Dawson 2001; Marino et al. 2010; Palumbi 1994; Pernet et al. 2008). Cytochrome C oxidase is a conserved gene, coding for the production of an enzyme involved in cellular respiration. Mutation rates of the COI gene are sufficient to detect nucleotide differences amongst individuals (Palumbi & Lessios 2004). This study employs a genetic analysis of COI gene nucleotide variations in collections of *Neotrypaea californiensis* larvae from the Oregon and Washington coast in 2005 and 2006 to examine temporal variation in the dispersing gene pool.

MATERIALS AND METHODS

Samples

Oceanic larval samples were harvested from June 2006 through September 2006 (Table 1) along both the Washington and Oregon coasts at hydrographic lines based on latitude ranging from La Push, Washington, to as far south as Cape Perpetua, Oregon. The lines employed within this study are off of Grays Harbor, Washington (GH); Willapa Bay, Washington (WB); the Columbia River (CR); Cape Meares, Oregon (CM); Cascade

Head, Oregon (CH); and Newport, Oregon (Newport Hydrographic, NH) (Figure 1).

Sampling took place at a distance between 1 and 15 miles offshore along these latitudinal lines. Plankton tows of the upper 20-30 m of the water column were made using a 330 μ m mesh bongo net system. Samples were preserved in 95% ethanol. Specimens were sorted for *N. californiensis* larvae which were measured and staged, and then placed in vials with 95% ethanol.

Additional samples were collected from within the Yaquina Bay (YB) estuary in Oregon, in July of 2006. Daily sampling of 100 to 120 m³ of water from the main tidal channel was done using a centrifugal plankton pump positioned off a dock at the Hatfield Marine Science Center. Zooplanktons were captured using a 350 μ m mesh plankton net, sorted and preserved.

Differentiation of the data set for a particular sampling year (2005, 2006) was made by addition of a suffix (05, 06) to the location (GH06, WB06, CR06, CM06, CH06, NH06, and YB06) (Table 1). Sampling efforts were supported by the Bonneville Power Authority, and the National Oceanic and Atmospheric Administration Fisheries Service as part of the Ocean Survival of Salmonids project.

Results of this analysis are compared to two associated studies (Doan unpublished data, Kozuka 2008). Kozuka's samples were collected along ocean lines in June, August and September of 2005 at the lines CR, CH, CM and NH (CR05, CH05, CM05, NH05), along with an additional line furthest to the south (Heceta Head, Oregon, HH or HH05) (Table 1). Michael Doan analyzed *N. californiensis* adult shrimp from within Yaquina Bay for the 2005 year (YB05).

Table 1. Sample collection dates and location.

Location	Position	Date of collection	Number sequenced	ID
Grays Harbor	46.91N, 124.30W	9/23/2006	5	GH06
Willapa Bay	46.68N, 124.30W	6/26/2006	4	WB06
Willapa Bay		9/24/2006	2	WB06
Columbia River	46.21N, 124.25W	6/19/2005	18	CR05
Columbia River		8/30/2005	59	CR05
Columbia River		6/24/2006	39	CR06
Columbia River		9/25/2006	7	CR06
Cape Meares	45.50N, 124.12W	6/20/2005	4	CM05
Cape Meares		8/31/2005	16	CM05
Cape Meares		6/26/2006	11	CM06
Cape Meares		9/26/2006	5	CM06
Cascade Head	45.05N, 124.20W	6/21/2005	60	CH05
Cascade Head		6/27/2006	6	CH06
Cascade Head		9/26/2006	3	CH06
Newport	44.62N, 124.25W	8/29/2005	10	NH05
Newport		6/28/2006	15	NH06
Newport		9/28/2006	3	NH06
Yaquina Bay	44.62N, 124.04W	7/14/2006	6	YB06
Yaquina Bay		7/19/2006	2	YB06
Yaquina Bay		7/26/2006	11	YB06
Yaquina Bay		7/27/2006	8	YB06
Heceta Head	44.13N, 124.20W	8/21/2005	5	HH05
Heceta Head		8/28/2005	5	HH05
Total:			304	

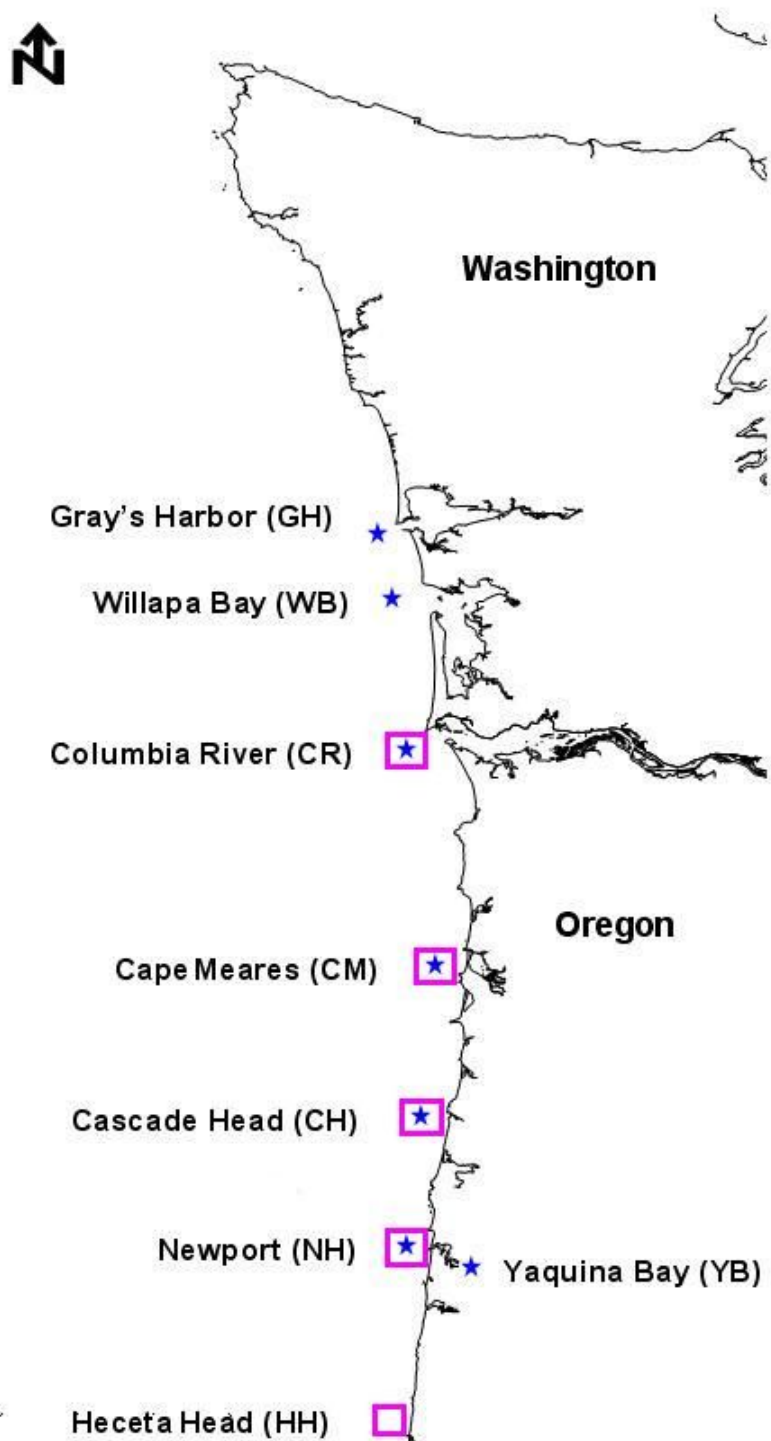


Figure 1. Map of sampling locations. Rectangles-locations at which planktonic larvae were collected in 2005. Stars-locations at which planktonic larvae were collected in 2006.

DNA extraction

DNA extraction from larvae followed methodology previously used within the Parr Laboratory (Kozuka 2008). Each larva was added to a mixture of 300 µl lysis buffer (0.5% SDS, 100 mM NaCl, 100 mM Tris [pH 8.0], 25 mM EDTA) and 100 µg of proteinase K (Fisher Scientific) to each sample. Samples were incubated at 65 °C for 0.5-2 hours until the tissue was fully digested. Incubation then continued for an additional 15 minutes at 37 °C upon the addition of 8 µg RNase (Fisher Scientific). Precipitation of proteins was performed with 7.5M ammonium acetate and the isolation of DNA with 100% isopropanol. At this point 10 µg of glycogen (Gentra Systems) was added to facilitate pelleting of DNA during centrifugation. The DNA was washed with 70% ethanol, air dried, then resuspended in 30 µl of TE buffer (10 mM Tris [pH 8.0] and 1 mM EDTA). Rehydration continued overnight. Samples were stored at 4 °C prior to PCR.

DNA amplification

A series of reamplification polymerase chain reaction (PCR) applications were used to amplify regions of COI, as used previously (Kozuka 2008). All PCR primers were designed using Primer3 v.0.3.0 software (Rozen & Skaletsky 2000). In order to amplify a 900-bp region of COI, PCR reaction was performed at a 25 µl reaction volume containing a buffered solution of 50mM KCl, 10 mM Tris [pH 8.3], 0.2 mM dNTPs (Fisher), 0.2 µM of the forward primer (SCOIFB 5' TGGGGCAATTACAATGTT 3') and 0.2 µM reverse primer (SCOIRB 5' ATCAGCAGGAGGATAAGGAT 3') with 0.4 mg/ml bovine serum

albumin (BSA), 4 mM MgCl₂, 1 unit Taq DNA polymerase (AllStar Scientific), and 10 ng larval DNA from extraction of tissue samples. A negative control was formulated by using sterile water in lieu of DNA. The PCR reactions took place in a Personal Thermal Mastercycler (Eppendorf) under the following parameters: initial denaturation for 5 minutes at 94 °C followed by 35 cycles of 30 seconds at 94 °C for denaturation, and then 45 seconds at 53-58 °C for primer annealing, and 1 minute at 72 °C for DNA strand extension. This was followed by a final extension step for 10 minutes at 72 °C. The nature of the amplicons was verified on a 2% agarose gel pre-stained with 1% ethidium bromide alongside an appropriate molecular size marker. The gel was run at 120-130 V for 45-60 minutes and visualized under ultraviolet light on a Bio-Rad Gel Doc unit.

A subsequent PCR reaction was performed as a nested PCR amplifying a 700-bp region within the 900-bp region amplified in the first reaction. Reaction volume totaled 25 µl, containing 0.2 mM dNTPs, 0.2 µM forward primer (SCOIFmore 5' TTTTGATCCAGCAGGAGGAG 3'), 0.2 µM reverse primer (SCOIRmore 5' GACCCTATAGAAGAAACCACATTTC 3'), 2 mM MgCl₂, and 0.5 unit Taq DNA polymerase. The amplicon resulting from the first PCR was diluted 10 to 1000-fold with water, and 1 µl of this dilution was used as the template. The concentration of DNA was estimated visually from the gel by comparison to the DNA marker run on the same gel. The thermal cycler parameters employed for this PCR were: 5 minutes at 94 °C followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 58-62 °C, and 1 minute at 72 °C followed by 72 °C for 10 minutes.

Subsequently a 591-bp region nested within the 700-bp region of the first nested PCR was amplified. It was generally necessary to follow these three steps to produce discrete fragment bands in sufficient quantity for sequencing. Thermal cyclers conditions were repeated as in previous nesting PCR reactions with the exception of forward primer (SCOIFnew 5' CCTGGGTTTGGTATAATTTCTCA 3') and reverse primer (SCOIRnew 5' ATCGGGGTAATCTGAATATCG 3'). Dilution of the amplicon was necessary to prevent nonspecific product from being synthesized during the reaction.

DNA sequencing

Samples that contained discrete fragment bands following gel electrophoresis were selected for sequencing. Excess dNTP's, primers, and single-stranded amplicons were removed by adding 2 µl ExoSAP-IT (USB) to bring to a total of 15 µl volume. Incubations for 30 minutes at 37 °C and 15 minutes at 80 °C were performed to ensure enzyme deactivation. The forward primer used for sequencing, COIFnew, was diluted to 5 µM. Samples were processed for sequencing at Geneway Research (Hayward, CA). Chromatogram sequences were obtained by use of an ABI Prism 3700 DNA Analyzer and automated sequencer (Applied Biosystems) using BigDye™ terminator methodology.

Data analysis

Chromatograms of a 548-bp region of the DNA were edited and aligned using the ClustalW multiple alignment algorithm in BioEdit software v7.0.9.0 (Hall 1999). After

manual editing to remove primer sequences, a 516-bp region was selected for comparison and use in all analyses.

The software package Mega 4.0 (Tamura et al. 2007) was employed to determine the amino acid sequence with comparison to a table of invertebrate mitochondrial DNA genetic code. A search was performed against the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) Protein database in order to identify similar sequences, and confirm the amplification of the targeted gene.

Nucleotide and haplotype diversity and measures of pairwise nucleotide distance were calculated using DnaSP 5.0 software (Librado & Rozas 2009). DnaSP 5.0 was also used to determine haplotype frequency and distribution.

Tests of population neutrality were conducted with Arlequin v3.11 software (Excoffier et al. 2005). Fu's F_S statistic (Fu 1997) indicates whether the level of diversity of a given sample (in terms of number of haplotypes present) is consistent with the number seen in a sample of equivalent genetic diversity that undergoes random mutation according to the Infinite Sites model. Tajima's D statistic was employed looking at segregating nucleotide sites in comparison to random mutation (Tajima 1989). The combination of the two tests examines whether the populations are selectively neutral and in equilibrium or under some selective pressure or demographic change. It is not entirely possible to disentangle possible demographic influences from those of selection on statistics.

Analysis of Molecular Variance (AMOVA) (Excoffier et al. 2005) is a measure employed to examine the genetic structure of a population utilizing an analysis of variance.

AMOVA was calculated on various collections with standard AMOVA methods and haplotypic format using Arlequin 3.11 software.

Fixation Index (F_{ST}) values, as assessed by AMOVA, of the overall and subpopulations were calculated with Arlequin software v3.11, with 1000 permutations, employing pairwise difference and calculating a distance matrix. F_{ST} examines the extent of genetic distance between subpopulations in comparison to the population as a whole (Bohonak 1999; Wright 1965). F_{ST} utilizes gene frequency and the number of mutations between haplotypes to estimate the pairwise divergence of haplotypes based on a distance matrix (Slatkin & Hudson 1991). F_{ST} values range from 0 indicating an individual population, to 1 indicating distinct populations. F_{ST} values were considered significant at p -values of less than 0.05 and highly significant at p -values of less than 0.001.

Relatedness was visualized using Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering method in Mega 4.0 (Tamura et al. 2007). F_{ST} distance values were used in the UPGMA tree construction among populations of larvae within the 2-year data set. UPGMA grouping assumes broadly that the rate of nucleotide or amino acid substitution is the same for all lineages. Branch lengths of the UPGMA dendrogram were calculated as half the distance between paired populations. It is not used in a phylogenetic sense strictly, as it does not infer common ancestral populations to extant population nodes. However, it is a useful dendrogram for grouping similar populations based on haplotype frequencies.

RESULTS

The combined 2005 and 2006 dataset exhibited haplotype (h) and nucleotide diversity (π) of ($h = 0.978$, $\pi = 0.043$) (Table 2). Haplotype and nucleotide diversity were given within both the 2005 ($h = 0.952$, $\pi = 0.040$), and the 2006 ($h = 0.984$, $\pi = 0.037$) individual data sets. The 177 larvae of the 2005 dataset had 104 distinguished haplotypes, with an average number of pairwise nucleotide differences of $k = 20.867$. The 127 larvae from 2006 had 87 distinguished haplotypes with an average number of pairwise nucleotide differences of $k = 19.114$. The 304 individual sequences of the combined dataset included 188 haplotypes with an average number of pairwise nucleotide differences of $k = 22.354$. The YB estuary larvae had a slightly lower haplotype diversity than larvae collected off shore ($h = 0.866$, $\pi = 0.038$). There were 63 variable sites within the 2006 dataset including 66 mutations. The 2005/2006 combined dataset included 80 variable sites with 85 mutations. The 2006 ocean larval population consisted entirely of silent substitutions, yielding an identical amino acid sequence within the analyzed region. The combined two-year dataset showed five replacement substitutions, changing expected amino acid sequence. Of these five replacement substitutions, four were found both in YB estuary samples from 2006 (YB06) and CH ocean larvae from 2005 (CH05).

Larval sequences from individual populations collected in 2006 had an average number of pairwise nucleotide differences ranged from a low of $k = 14.400$ (GH06) to a high of $k = 19.610$ (YB06).

Table 2. Haplotype (h) and diversity (π) indices. k = mean number of pairwise nucleotide differences. n = number of sequences

ID	h	π	k	n
GH06	0.900	0.028	14.400	5
WB06	1.000	0.031	15.933	6
CR05	0.951	0.036	18.433	77
CR06	0.981	0.031	15.948	46
CM05	0.974	0.036	18.758	20
CM06	1.000	0.027	13.967	16
CH05	0.954	0.048	24.817	60
CH06	0.972	0.037	18.944	9
NH05	0.933	0.018	9.444	10
NH06	0.974	0.035	18.163	18
YB06	0.866	0.038	19.610	27
HH05	0.867	0.021	10.978	10
2005	0.952	0.040	20.867	177
2006	0.984	0.037	19.114	127
All	0.978	0.043	22.354	304

Haplotypes were compared both within and among the two years of the data set (Table 3). The single most common haplotype (H1) occurred within the 2005 ocean sample set ($n = 33$). All of these samples were harvested off of the Columbia River in

2005 (CR05). This haplotype contains samples collected both in the spring ($n = 12$) and fall ($n = 21$) of 2005 (Table 1). In the two-year data set, the most common haplotype (H1) was shared by 33 of the total 304 individuals, all of these were from 2005. The second and third most common haplotypes (H2, H3) were identified in both years of the study. There were no further haplotypes spanning both years. Of the 137 individuals that shared a haplotype with at least one other individual, 57 are from CR. Of the 27 larval samples taken from the YB estuary in 2006 (YB06), 10 shared a single haplotype (H3). This haplotype was also found in five samples from the previous year, offshore to the north (CH05). Haplotypes consisting of a single individual (singletons) numbered 169, or 55.59% of all larval samples. There were no universal haplotypes shared among all of the populations in the combined two-year data set (Table 3).

Neutrality statistics for both yearly data sets showed significant negative values for Fu's F_S statistic. 2005 data showed an F_S value of -23.749 ($p = 0.006$) and 2006 data showed an F_S value of -23.903 ($p = 0.000$) (Table 4). The combined two-year dataset showed an F_S value of -23.547 ($p = 0.006$). When individual populations were examined, F_S reached significance at only one subpopulation, CM06 ($F_S = -6.491$, $p = 0.006$).

Tajima's D test of neutrality (Table 4) was significant ($p > 0.95$) at $D = 1.547$ for 2005 and at $D = 2.026$ for the 2006 dataset. The combined two-year dataset was also significant at $D = 2.282$.

When individual populations within the 2005 or 2006 dataset were examined, five (CR05, CR06, CM05, CH05, NH06) yielded a significant result under Tajima's D statistic

($p > 0.95$). The remaining seven populations did not yield significant results, and two showed negative values. These were spread among both yearly collections.

Table 3. Haplotype distribution and composition (where $n > 2$). Ocean 05 = ocean larval subpopulations of 2005. Ocean 06 = ocean larval subpopulations of 2006. YB Adults 05 = Yaquina Bay adults samples of 2005.

Common Haplotypes ($n > 2$)										
ID	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
GH06									2	
WB06								1		
CR05	33	9		7	7					
CR06		3				3	5	2	2	2
CM05		1			2					
CM06							1	1		1
CH05		2	5	6	2					
CH06		2								
NH05										
NH06		1				3		2		
YB06			10			1				
HH05										
Total	33	18	15	13	11	7	6	6	4	3

Ocean 05	33	12	5	13	11					
Ocean 06		6				6	6	6	4	3

YB Adults 05	11	5			14					
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Table 4. Tests of neutrality. Bold values indicate significance (Tajima's D , $p > 0.95$; and Fu's F_S , $p < 0.02$).

ID	Tajima's D	Tajima's D p -value	Fu's F_S	F_S p -value
GH06	1.146	0.859	2.209	0.785
WB06	0.251	0.603	-0.290	0.240
CR05	1.605	0.951	-9.125	0.034
CR06	1.820	0.978	-9.124	0.011
CM05	1.552	0.970	-1.359	0.263
CM06	0.548	0.760	-6.491	0.006
CH05	2.241	0.991	-4.103	0.162
CH06	1.297	0.931	0.192	0.435
NH05	-0.664	0.271	-0.435	0.338
NH06	1.592	0.974	-1.579	0.201
HH05	-0.886	0.215	1.093	0.674
YB06	1.158	0.915	1.247	0.743
2005	1.547	0.951	-23.749	0.006
2006	2.026	0.979	-23.903	0.000
All	2.282	0.980	-23.547	0.006

Genetic distance between populations of the combined 2-year data set, examined as F_{ST} values, yielded many significant results (shown with asterisks in Table 5). The F_{ST} data from 2005 shows the CH05 population was significantly different from two (NH05, HH05) of other four populations from that year ($p < 0.05$). The only other significant difference was between the HH05 and CM05 populations ($p < 0.05$). F_{ST} values from 2006 were highly significant ($p < 0.001$) between the YB06 estuary samples and four of

the six remaining ocean populations (CR06, CM06, CH06, and NH06). The GH06 and WB06 populations showed a significant difference ($p < 0.05$) with YB06. NH06, the nearest ocean population in proximity to YB, showed highly significant restricted gene flow between itself and YB06 ($F_{ST} = 0.190$, $p = 0.00$). There was no significant genetic distance evident in any of the 2006 ocean larval populations as none of the F_{ST} values reached significance (Table 5).

In comparing populations that occur in both 2005 and 2006 data, there were highly significant ($p < 0.001$) differences from one year to the next between three of the four collections (CR, CM, NH). CH was the only population that did not show a significant difference ($p = 0.28$) over the two years of collection.

The largest subpopulation, CR, contained samples from both 2005 and 2006. In addition CR05 and CR06 contained samples from both spring and fall sampling. Comparing CR05 spring samples with CR05 fall samples showed a highly significant F_{ST} of 0.53 ($p = 0.00$). Comparison of CR06 spring samples against those from CR06 fall showed a significant F_{ST} of 0.16 ($p = 0.004$).

Table 5. Pairwise F_{ST} values and significance as assessed by AMOVA in 2005 (05) and 2006 (06). (* = $p < 0.05$; ** = $p < 0.001$). WB = Willapa Bay, GH = Gray's Harbor, CR = Columbia River, CM = Cape Meares, CH = Cascade Head, NH = Newport, YB = Yaquina Bay, HH = Heceta Head.

ID	GH06	WB06	CR05	CR06	CM05	CM06
GH06	0					
WB06	0.05601	0				
CR05	0.34405**	0.19918	0			
CR06	0.08948	-0.04114	0.20101**	0		
CM05	0.35484**	0.19966*	0.03069	0.21694**	0	
CM06	0.10918	-0.0767	0.2278**	-0.01642	0.2409**	0
CH05	0.16399*	0.09554	0.06074	0.12058	0.09295	0.13905*
CH06	0.12681	0.05457	0.08639*	0.05244	0.10961*	0.09293
NH05	0.61957**	0.46589*	0.04297	0.38251**	0.08117	0.46406**
NH06	0.0101	0.02743	0.20126**	0.02458	0.21655**	0.05738*
HH05	0.57498**	0.38867**	0.05093	0.32899**	0.11994*	0.40346**
YB06	0.16976*	0.21568*	0.40869**	0.26851**	0.41297**	0.26932**
YB05 Adult	0.79242**	0.69173**	0.16717**	0.55163**	0.31173**	0.66291**

ID	CH05	CH06	NH05	NH06	HH05	YB06
GH06						
WB06						
CR05						
CR06						
CM05						
CM06						
CH05	0					
CH06	0.01067	0				
NH05	0.1455*	0.27813**	0			
NH06	0.07846*	-0.00036	0.39663**	0		
HH05	0.13349*	0.25586**	0.0312	0.36163**	0	
YB06	0.20382**	0.2424**	0.53972**	0.19022**	0.5189**	0
YB05 Adult	0.29225**	0.54341**	0.12508*	0.67072**	0.20469**	0.70411**

Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering method was used to visualize a dendrogram calculated on the basis of distance values (F_{ST}). Branches are considered significant at F_{ST} values greater than 0.05. The UPGMA dendrogram of the 2005 oceanic larval collections supported a three-way grouping of the five sites based on latitude (Figure 2). The CH05 population offered a central branch positioned between a northerly branch consisting of CR05 and CM05 populations and a southerly branch containing NH05 and HH05 populations. This grouping did not occur in the 2006 UPGMA dendrogram (Figure 3). There was some evidence of a north-to-south grouping within the 2006 tree. GH06, being the most northerly of the six ocean populations, formed its own branch. The other five populations formed two separate branches showing little differentiation from within. WB06, CR06 and CM06 formed one branch which contains adjacent geographical populations. The other branch formed a more southerly grouping of CH06 and NH06 populations. However, it should be reiterated that the separation between these three branches was not significant. The YB06 population was always the most distantly separated from other populations, as shown in the 2006 UPGMA (Figure 3), and years-combined UPGMA diagrams (Figure 4).

When comparing the two yearly populations in single dendrogram, the patterns mentioned above were maintained (Figure 4). A pattern of three significant groups was formed; the 2005 ocean larvae, the 2006 ocean larvae, and the 2006 YB estuary larvae. These three groups were also examined as a whole by F_{ST} pairwise distances, and all comparisons between the three populations showed high significance ($p = 0.000$) (Table 6)

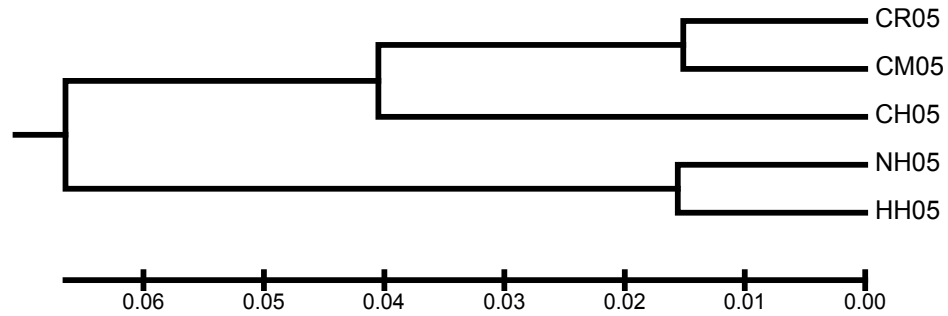


Figure 2. UPGMA dendrogram of F_{ST} distances among groups of larvae collected in different areas in 2005 (05). CR = Columbia River, CM = Cape Meares, CH = Cascade Head, NH = Newport, HH = Heceta Head.

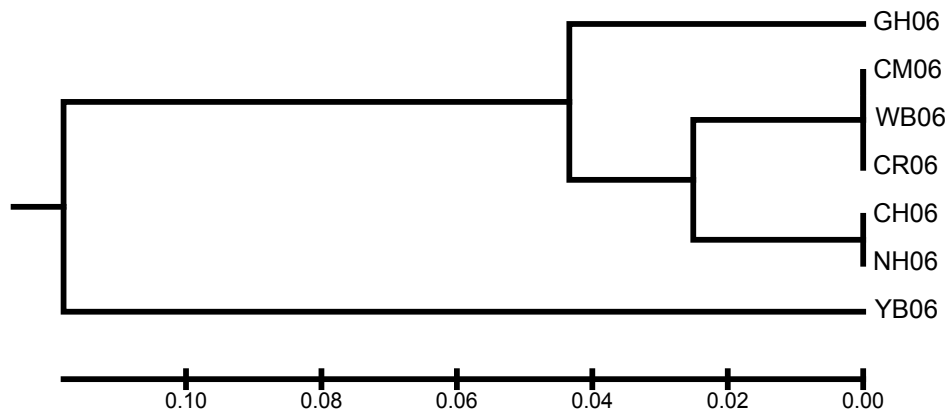


Figure 3. UPGMA dendrogram of F_{ST} distances among groups of larvae collected in different areas in 2006 (06). WB = Willapa Bay, GH = Gray's Harbor, CR = Columbia River, CM = Cape Meares, CH = Cascade Head, NH = Newport, YB = Yaquina Bay.

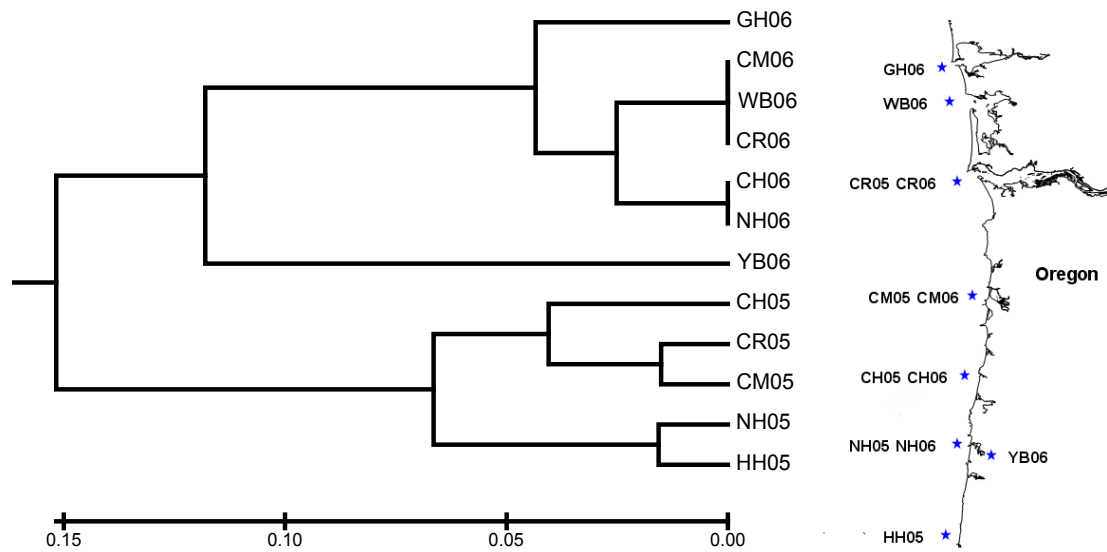


Figure 4. UPGMA dendrogram of F_{ST} distances among groups of larvae collected in different areas in both 2005 (05) and 2006 (06). WB = Willapa Bay, GH = Gray's Harbor, CR = Columbia River, CM = Cape Meares, CH = Cascade Head, NH = Newport, YB = Yaquina Bay, HH = Heceta Head.

Table 6. Pairwise F_{ST} values and significance as assessed by AMOVA. Oceanic larvae of 2005, Oceanic larvae of 2006, and Yaquina Bay larvae of 2006). (* = $p < 0.05$; ** = $p < 0.001$).

	Ocean 2005	YB 2006	Ocean 2006
Ocean 2005	0		
YB 2006	0.40191**	0	
Ocean 2006	0.1605**	0.31914**	0

The AMOVA performed on three groupings of populations: ocean larvae from 2006, ocean larvae from 2005, and the YB estuary larvae from 2006 (Table 7) supported significant differentiation among the three groupings. Minimal variation was shown within

the three groupings (percentage of variation = 2.69%), with the majority of variation being represented within the populations as a whole (percentage of variation = 70.93%).

Table 7. Parameters and test statistics analyzed by AMOVA. (= p -value < 0.001)**

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	2	226.951	3.762 Va	26.38
Among groups within populations	5	122.726	0.384 Vb	2.69
Within populations	286	2895.449	10.123 Vc	70.93
Total	293	3245.126	14.273	
Group 1= 2005 Ocean Larvae			F_{SC} :	0.0366**
Group 2 =2006 Ocean Larvae			F_{ST} :	0.29071**
Group 3 =2006 YB Estuary Samples			F_{CT} :	0.26376**

The AMOVA comparing the oceanic larvae of 2005 and those of 2006 (Table 8), showed results similar to those of the three-way grouping, with minimal variation being shown within the two groupings (percentage of variation = 4.75%), and the majority of variation represented within the population as a whole (percentage of variation = 80.18%).

Table 8. AMOVA Oceanic larval groupings only (excluding YB) (= p -value < 0.001).**

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	251.807	1.744 Va	15.06
Among groups within populations	9	188.637	0.550 Vb	4.75
Within populations	265	2459.818	9.282 Vc	80.18
Total	275	2900.261	11.576	
Group 1= 2005 Ocean Larvae			F_{SC} :	0.05595**
Group 2 =2006 Ocean Larvae			F_{ST} :	0.19817**
			F_{CT} :	0.15065**

DISCUSSION

We expected to find evidence of strong widespread gene flow and a lack of significant genetic distances between populations of *N. californiensis* larvae that were sampled offshore. This is according to the expectation that long-lived larvae developing for six to eight weeks (Dumbauld et al. 1996) would be dispersed widely by the highly active currents off the coast of Oregon and Washington. The assumption here was that high flow rates of larvae in the California Current would result in net larval dispersal to the south and out of the immediate sampling range. This would leave little evidence of significant gene flow from the previous season's dispersal event, as haplotypes would

more often enter southern sites. The assumption was also made that the lack of barriers to gene flow within ocean populations sampled in 2005 would be confirmed by subsequent sampling during the 2006 season.

The haplotype diversity found within both the 2005 and 2006 seasons' samplings indicated a large percentage of the haplotypes were unique and not shared among individuals (singletons). These findings are in accordance with a sudden expansion scenario (Slatkin & Hudson 1991). This also supports a highly reproductive population with widely dispersing larvae (Dawson 2001).

Neutrality test results from Fu's F_S supported an excess of rare alleles within the population leading to a proliferation of haplotypes (Fu 1997; Tajima 1989). The possible explanations for the findings range from a recent demographic expansion to selective pressure. A reduction in population size or a balancing selection could be occurring. Examining individual subpopulations by Tajima's D show that there is a possibility of certain polymorphisms being over-represented, such as in a founder effect.

Four of five ocean populations that were sampled across each of the two years of this study showed a genetic distance that is highly significant based on F_{ST} values (Table 5). There were 35 pairwise combinations of populations that could be compared between 2005 and 2006. Genetic distance was significant in 31 of these comparisons. This supports the inference that from one year to the next there was a rapidly changing population of larvae present off of the coast.

An examination of larvae sampled at the Columbia River within ocean waters (CR05) showed significant genetic distance between samples taken during spring, early in

the dispersal season, and later into the fall, when recruitment to estuaries occurs. This observation is further corroborated by similar results in the next year (CR06). Therefore, genetic distance between populations seems high not only from year to year but also between cohorts of spring and fall recruitment.

The examination of samples within the 2006 set of ocean larvae revealed no evidence of either restricted gene flow or geographic barrier to dispersal. This was consistent with data of the previous year's (2005) ocean sampling despite the fact that the 2006 population was extended to cover a larger range (extending to the north, into southern Washington).

The larval samples taken in 2006 from within Yaquina Bay (YB06) showed weak gene flow in all comparisons to offshore populations within the same year. This was also true when the estuary larvae were compared to the previous year's ocean population. All comparisons showed significant genetic distance between the estuary and ocean populations. F_{ST} values of the Yaquina Bay population compared against either year's ocean populations were highly significant (Table 5), indicating a lack of mixing. Yaquina Bay samples also were differentiated from larval samples collected at the nearest offshore population which was Newport (NH), located only 1-10 miles from the mouth of the Yaquina Bay estuary. It must be noted that the time period of ocean sampling does not coincide directly with the estuary sampling. The variation was as much as one month prior and two months following the estuary collection.

Haplotype diversity within the YB sampling was lower than within either yearly ocean population. Haplotypes from the YB population had the highest percentage of a single common haplotype and lowest percentage of singletons.

These observations suggest recruitment of larvae from nearby offshore populations to within the estuary was not as extensive as, or may have been more complex, than previously supposed. Larvae may have been entrained (following release from local adult populations or entry from the ocean) for an extended period. Any tendency toward enclosed populations has been shown to increase phylogeographic structure (Dawson 2001).

Adult *N. californiensis* sequences from an associated study within Yaquina Bay during 2005 (Doan unpublished data), showed no common haplotypes and a significant genetic distance with the larvae from within YB in 2006 (Table 5). This does not agree with the previous inference of an entrained population.

The data indicates that larval dispersal and recruitment was complex and varied in time. The most common haplotype from within YB larvae of 2006 (H3) was also among the most common haplotypes found in one of the ocean populations off of Cascade Head in the previous year (CH05). However, these two subpopulations showed low gene flow ($F_{ST} = 0.204$) (Table 5). Yaquina Bay adult shrimp from 2005 and YB larvae from 2006 did not share any haplotypes. However, they each shared haplotypes with several ocean subpopulations to the north (Table 3). In pairwise comparisons of F_{ST} values, the YB adult population of 2005 showed a highly significant genetic distance as compared to all ocean populations and to the YB 2006 larvae (Table 5).

It is not clear what was occurring in the exchange of YB larvae with ocean populations and subsequent recruitment. Another possibility is that the estuary samples dispersal was largely to ocean populations not within the sample area, perhaps farther to the south along the dominant offshore current. There is however no evidence in this study that directly supports this inference.

Distribution of zooplankton from various species throughout Yaquina Bay has been previously studied (Frolander et al. 1973). Larvae found within the estuary during the summer months were predominantly from adults that were found in highest density along coastal areas to the north of Yaquina Bay. This pattern corresponded to the prevailing southerly flow of the California Current during the same time frame. Larvae found within the estuary during winter months were predominantly from species with adults located to the south of Yaquina Bay. This supports the inference that many larvae collected during this study from within Yaquina Bay would likely have dispersed to the south as well. As a result, this would have left them outside the sample area, which was primarily to the north of Yaquina Bay.

A recent study of the Mediterranean Shore Crab, *Carcinus aestuarii*, examined populations in the Venice Lagoon of Italy (Marino et al. 2010), and suggests another plausible explanation for the significantly different larval and adult sampling within YB. The presence of significant differences on a micro-geographic scale within the YB estuary may be due varying selective pressures at these different sites. This is also suggested by other studies (Barber et al. 2002; Mackie et al. 2009). Sampling methodology could have

therefore led to a significant difference in genetic distance depending on location within the estuary. There is also evidence that this pattern was variable from year to year.

However, data in the present study does not suggest selection is the simplest explanation for why there would be site-to-site mitochondrial DNA differences in *N. californiensis* populations. The fact that oceanic larval pools adjacent to estuaries may differ in genetic composition from one year to the next (as was shown in the 2005 and 2006 samples compared here), suggests that randomness in where particular sets of larvae are moved into estuaries could in fact explain much of the genetic differences seen in the estuary populations.

It may be that genetic differentiation within estuaries is absent. Analysis of sites within 20 estuaries in the Oregonian region (Parr and Mackie, unpublished data) supports a general lack of within-estuary genetic differentiation among adults. The Yaquina Bay adult samples of 2005 (YB05) did not show significant genetic distance among any of the three sample locations throughout the estuary. However larval samples of the following year (YB06) do show highly significant genetic distance to those of 2005 adults, though sampled just across the estuary (Figure 5).

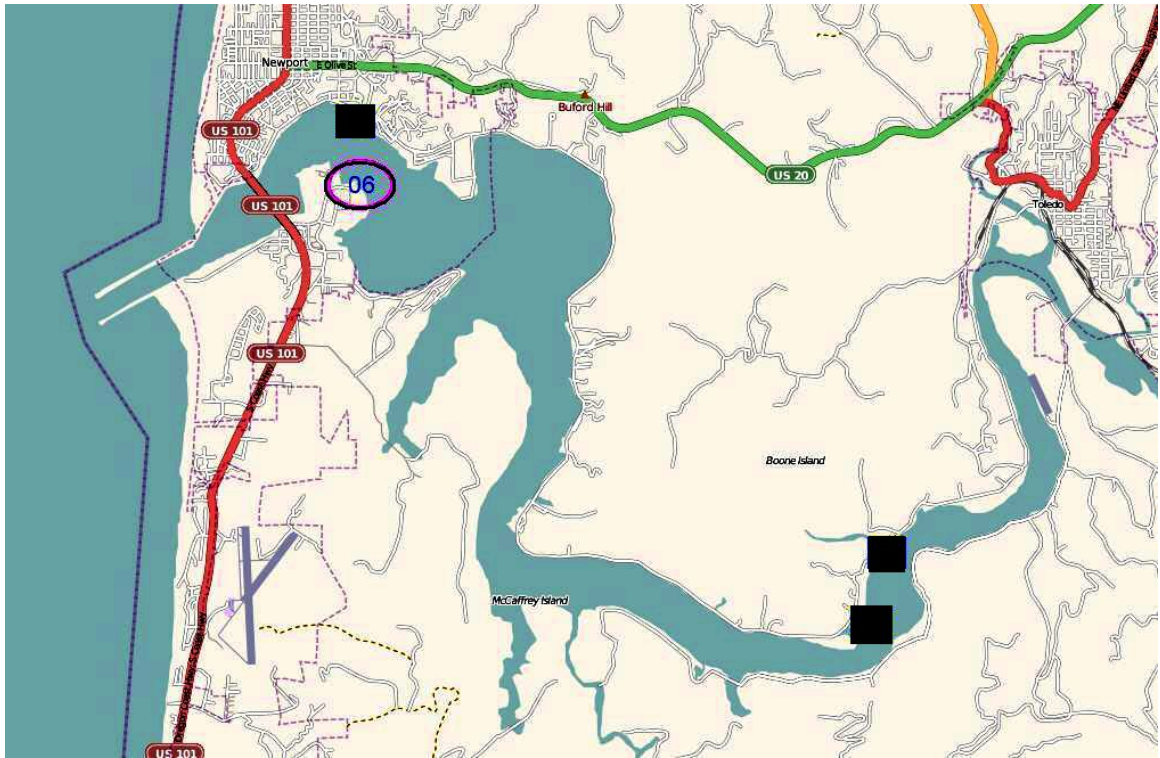


Figure 5. Yaquina Bay sample locations. Rectangle = 2005, Oval = 2006. Map data (c) OpenStreetMap (and) contributors, CC-BY-SA.

The Yaquina Bay larvae sampled and sequenced as part of this study were all classified as stage I, which has been established to correspond to an age of ten days or less (Cassidy 2009). Therefore it is likely that all of these larvae were released in near proximity to the sample site and form a comparatively homogeneous group. This is supported by the genetic analysis within this study. In addition, all of the YB06 larvae were sampled within a thirteen day period of July 2006 (Table 1). It is possible that COI variation between the YB05 adults and the YB06 larvae is simply due to a recent larval release by a small group of adults near the YB06 sampling site. The lack of any temporal variation in sampling, does not allow for a complete picture of the larval population within

YB. It is known that shrimp larvae may be released over any of a number of months in this species (Dumbauld et al. 1996). This may also exacerbate the statistical differences between the estuary larvae and the ocean larvae, especially in light of the fact that the ocean samples were collected over a range of several miles while the YB06 samples were collected in a single location. The fact still remains that both the larval and adult samples within this study form a highly genetically distinct group, both in comparison to each other, and to offshore populations.

Examination of ocean currents within the sampling area for the sampling period may offer insight into nature of larval retention. It has been shown that both the velocity and direction of localized currents within the immediate sampling area vary greatly over the sampling period from 2005 to 2006 (Kosro 2006; Kosro et al. 2006). Velocities at times reach as high as 80 cm/s at locations near our sampling sites. This would lead to rapid larval dispersal. Specifically, when examining current rates in July off the coast of Newport, Oregon, there were significantly higher velocities toward the southwest in late July of 2005 than in July of 2006 (Figure 6). This corresponds to the time frame of YB06 larval sampling from within the estuary.

Evidence of the effect of strong deviations in current flow during the 2005 season on the resulting recruitment of several other species along this coastline has been reported by Barth et al. (2007). Wind and temperature alterations significantly delayed early season (April through June) and intensified late season (August through September) upwelling currents. This was shown to significantly alter recruitment patterns of barnacles and mussels at sampling stations that are also within the sample area of this study. Ocean

currents and conditions varied significantly over the 2005-2006 seasons covered by this study. These data support the conclusion that the journey of larvae would vary greatly from one season to another, and perhaps particularly in 2005-2006.

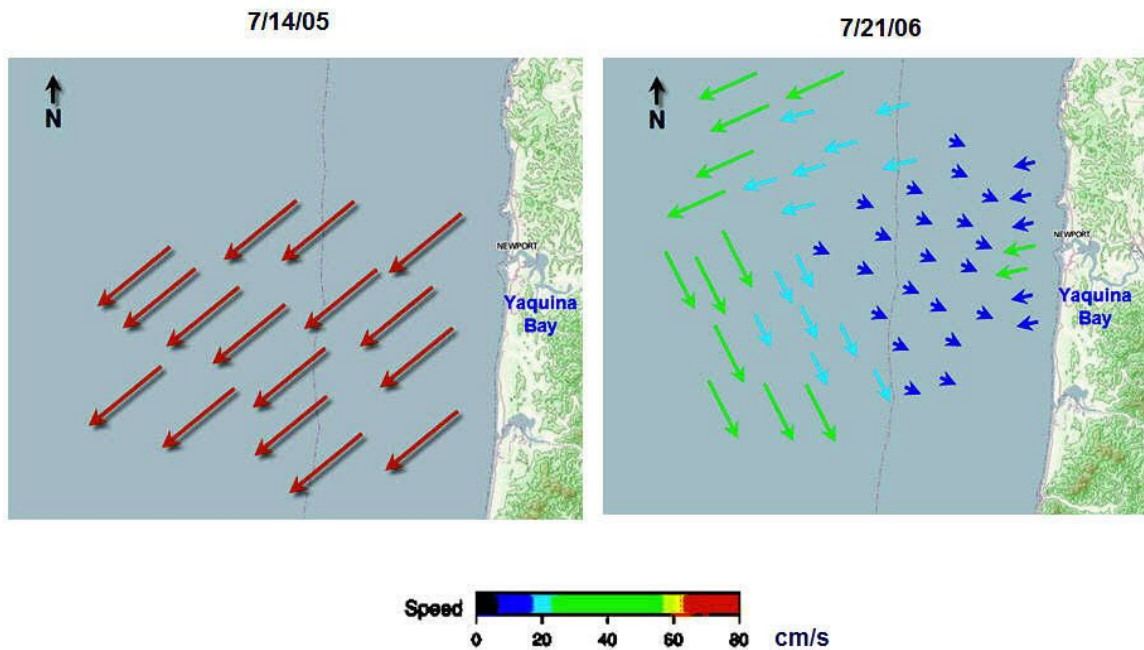


Figure 6. Ocean currents off Newport OR, July 2005 and July 2006. Darker shades = Higher velocity (Kosro 2006).

Variation in larval release times over a number of months by adults within the estuary may lead to distinct genetic groups exiting to the ocean at any one time. According to this explanation, genetic drift may play a major role in determining population diversity. It can be expected that long planktonic duration within the ocean currents would lead to a mixing of such groups over a number of months. A genetically homogenous ocean population at any time may lead, through a variety of causes (larval

release timing, ocean currents, and possibly post-settlement selection) to a highly structured population within an estuary such as Yaquina Bay (Marino *et al.* 2010). Temporal variation in ocean borne larval pools over the course of a season or several years, would contribute to this further. An interesting and important question in terms of population maintenance is how density of recruitment varies from year to year. Current evidence suggests that recruitment levels differ greatly from year to year (Barth *et al.* 2007).

This study shows that there was significant genetic distance between the populations of one year (2005) to those of the subsequent year (2006) in a benthic estuarine crustacean, *Neotrypaea californiensis*. We found no evidence of a significant barrier to gene flow along the portion of coastline included in this study, within either of two sampled years. Yaquina Bay estuary samples had significant genetic distance from nearby drifting oceanic larvae, and other adult populations (Parr and Mackie unpublished data), suggesting that overall, gene flow among even neighboring estuaries was limited. One interpretation is that larvae may have been entrained within the estuary and under a variety of selective pressures. An alternative possibility is that if any dispersal was occurring, these larvae may have been evicted from the sample area quickly. As a result, any subsequent recruitment to estuary populations would have arrived from sources somewhat distant geographically. Therefore if any of the Yaquina Bay estuary larvae within this study reached the adult reproductive stage, they would have likely done so at a location different from their natal site. These adults would therefore not have contributed greatly to the larval population found off of the coastline sampled in this study. Site-

specific differences in natural selection would not have evolutionary effect, because due to oceanic current activity and lack of philopatric dispersal mechanisms, populations in different estuaries may tend to become outbred over many years, as different sets of larvae arrive in different years.

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